EXPERIMENTS ON THE NORMAL MEIOTIC BLOCK IN THE OVUM OF TRITURUS VIRIDESCENS[1]

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At the time of penetration of the amphibian egg by the sperm, the second division of meiosis is arrested at metaphase. Entrance of the sperm triggers the activation of the blocked division and the second polar body is produced. Much interest has, through the years, been centered about the problem of just what triggers the completion of the division as a part of the "activation" of the egg. Equally fascinating, but largely ignored, is the question of the nature and origin of the meiotic block which is removed by the entrance of the sperm.

The variation in the stage of meiosis customarily reached by the eggs of the major animal groups before fertilization has been known for many years (for references see Wilson, 1925; Just, 1939; Dalcq, 1957; Rothschild, 1956). In the vertebrates, the egg is usually fertilized at metaphase of the second maturation division, and it is commonly stated that the division in unfertilized eggs is blocked at this stage. This statement holds for most amphibian eggs, although variations have been noted for *Siredon* (*Ambystoma*) (Schultze, 1887) and for *Triturus viridescens* (Humphries, 1955, 1956). As will be shown in this paper, the exceptions in *T. viridescens* seem to occur only under certain circumstances.

In earlier work (Humphries, 1956) it was shown that in *Triturus viridescens* the first maturation spindle forms at about the time of ovulation, and the first polar body is extruded soon after the egg enters the oviduct. The second division figure then forms and remains at metaphase until fertilization occurs. Coelomic eggs which, for any reason, are delayed in entering the oviduct, extrude the first polar body in the coelom and show the formation of the second metaphase figure in the same way as do oviducal eggs. However, two of the coelomic eggs in that study had passed the usual stage of arrest and were in anaphase II. It seemed possible that the difference between the oviducal and coelomic environments might be responsible for the escape from the block in these eggs and, further, that the oviducal environment might be a factor in the normal blocking of meiosis in unfertilized eggs. The present report deals with an investigation of these possibilities, using two approaches: (1) ligation of the oviducts at several levels, to retain the eggs abnormally long in the coelom or in certain regions of the oviducts, and (2) maintenance of eggs *in vitro*, with and without jelly coats.

Materials and Methods

Ligation experiments: Adult *Triturus viridescens* females were obtained from ponds near Charlottesville, Virginia, and Franklin and Canton, North Carolina,

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and kept in a refrigerator at about 12° C. until used. The oviducts in this species show a gross differentiation into distinct regions (Adams, 1940) which have been identified, from anterior to posterior, as regions A through E (Humphries and Hughes, 1959). An ostial portion, which is apparently non-secretory, precedes region A. The deposition of jelly about the egg begins as the egg passes through region A. The oviducts were ligated as close to the ostium as possible, or at the posterior limits of the A, C, or D regions. Following ligation, the females were allowed to recover for a period of a few days to two weeks, after which they received a subcutaneous implantation of one or two intact anterior lobes of the pituitary of *Rana pipiens*. If two lobes were used, the second implantation followed the first by 48 hours. Animals were sacrificed and opened 48 or 72 hours after the final pituitary implantation and any eggs which had accumulated in the coelom or oviducts were removed, placed in Bouin's fixative, embedded in Tissuemat, sectioned at 10 microns, and stained with Harris' haemalum and fast green. In the first experiments done, using only animals with oviducts ligated at the ostium, four advances past metaphase II were obtained from a total of 66 eggs which had reached at least metaphase II. Two were from "48-hour animals," and two were from "72-hour animals." It apeared, therefore, that 48 hours was an adequate time interval in which to expect advances and this was used for all remaining ligation experiments. The eggs used in these experiments were obtained from a total of 43 animals. Statistical analysis was made through the use of the contingency chi-square (Mather, 1943), using the Yates correction.

In vitro experiments: Animals used in this part of the study were obtained from lakes near Franklin and Highlands, North Carolina. In this group ovulation was stimulated by the injection into the coelom of 0.25 to 0.40 cc. of chorionic gonadotropin (Antuitrin "S," Parke, Davis, 500 units per cc.). When necessary, a second injection was given, usually 48 hours after the first. Eggs present in the coelom, ostium, and secreting parts of the oviducts were removed following decapitation of the animals, and were placed in solutions in covered syracuse dishes, the bottoms of which, in most instances, had been covered with paraffin to prevent adhesion of eggs to the glass. In some cases, the bottom was covered with filter paper wet with one of the solutions used. About a third of the oviducal eggs were freed mechanically from their jelly, using watchmaker's forceps, before being placed in solution. The solutions employed were Holtfreter's solution, Ringer's solution, and Niu-Twitty solution. Some eggs were kept in small droplets of solution, barely sufficient to cover them, while others were kept in well filled dishes. In the case of eggs kept in droplets, and in the case of those kept on wet filter paper, the covers of the dishes were sealed with Vaseline. Most of the eggs were kept at 20° C. in a water bath for 24 hours, though some were kept for 12 or 48 hours. Fixation and processing were the same as outlined for the previous experiments, except for a few eggs which were stained in toluidine blue for cytochemical study. The eggs used in this part of the study were taken from a total of 49 animals.

The statistical method used was the same as for the results of the ligation experiments.

Observations

Ligation experiments: The stages of meiosis in eggs of the various categories are given in Table I. Ovulation occurs over an extended period in this species;

thus, some of the eggs were probably out of the ovary and in the coelom or oviducts for two days or more prior to fixation, while others may have been released from the ovary only a few minutes before the animal was sacrificed. There is no way of knowing, other than in a general way, based on position in the oviduct and stage of meiosis, just how long a given egg has been out of the ovary. Under the circumstances, one would expect to find the entire spectrum of stages of meiosis, ranging from metaphase I through metaphase II and perhaps beyond. Such a situation is reflected in the distribution shown in Table I.

TABLE I

Stages of meiosis in oocytes removed from coelom and oviducts of operated animals 48 or 72 hours following final pituitary implantation*

Source	Earlier than Metaphase II	Metaphase II	Anaphase II to Telophase II	Meiosis complete
Coelom—ovid. not ligated at ostium	43	81	0	0
Coelom—ovid. ligated at ostium	32	44	0	1
Ostium—ovid. ligated in ostial region	17	35	1	3
Region A—ovid. ligated post. end of "A"	7	14	0	0
Region A—ovid. ligated post. end of "C"	1	10	0	0
Region A—ovid. ligated post. end of "D"	1	0	0	0
Region B—ovid. ligated post. end of "C"	1	0	0	0
Region C—ovid. ligated post. end of "C"	3	167 (51 had no jelly)	0	2**
Region D—ovid. ligated post. end of "C"	1	3	0	0
Region D—ovid. ligated post. end of "D"	2	38	0	0
Coelom—escaped from oviduct—jelly-covered	1	58	0	0
Totals	109	450	1	6

* See text.
** Not jelly-covered.

Meiosis was judged to be complete under one of the following conditions: presence of two polar bodies; presence of two polar bodies plus a single set of single chromosomes (monads) within the egg (Fig. 3); presence of a set of monads within the egg, in the absence of one or both polar bodies. In these eggs the single chromosomes within the egg were never in other than the condensed condition of mitotic chromosomes, nor were they surrounded by a nuclear membrane. In the one case of anaphase II which was observed, single chromosomes were about half-way to the poles and the first polar body was present (Figs. 1 and 2).

Meiosis was found to be advanced or complete only in eggs with no jelly coats. Advances occurred in coelomic eggs, in eggs from the ostial (non-jelly-secreting)

region of the oviducts, and in non-jelly-covered eggs from region C of the oviducts. Region C is a secreting region, and eggs in this region ordinarily have jelly coats deposited by the C region and by more anterior parts of the oviduct, but in a few instances the ligated oviducts became so distended with eggs that eggs which were

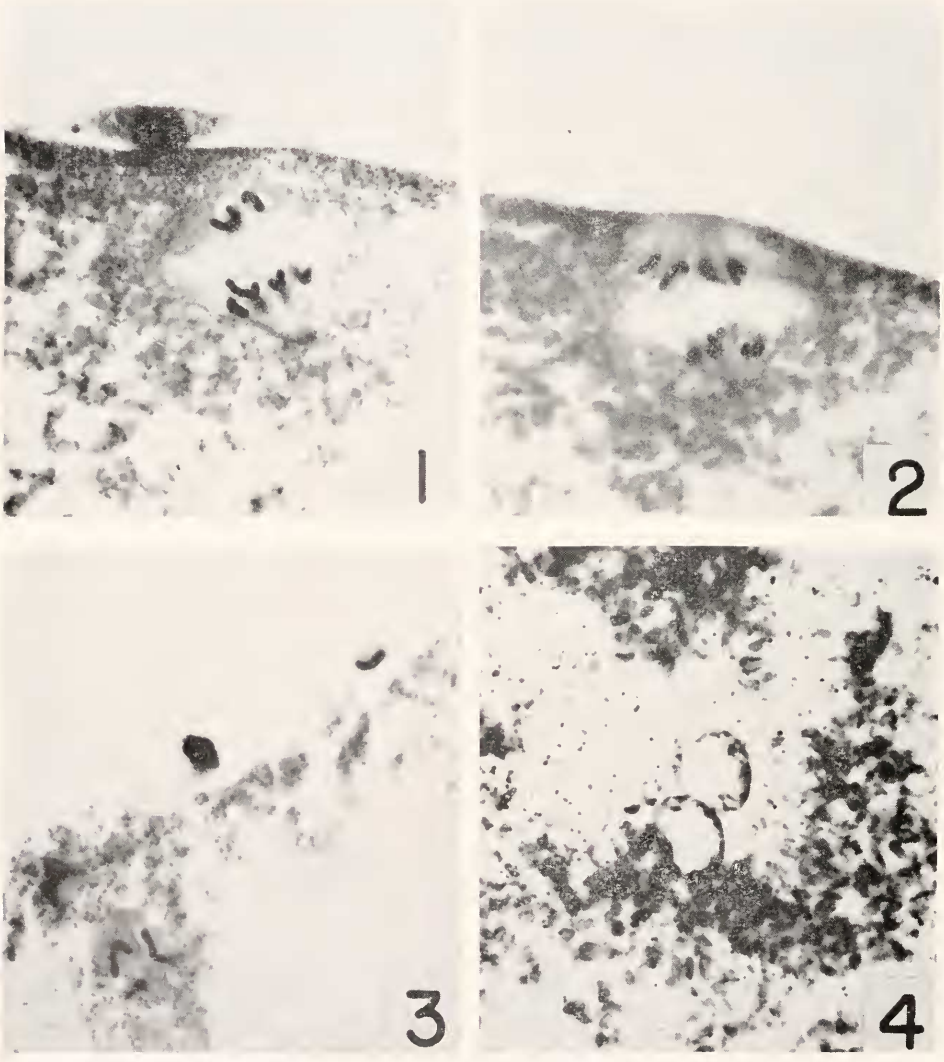

FIGURE 1. Anaphase II with adjacent polar body I, from the ostium of an oviduct ligated as close as possible to the anterior end. × 790.

FIGURE 2. Section adjacent to that shown in Figure I. × 790.

FIGURE 3. Completed meiosis in an egg from the coelom of an animal with oviducts ligated at the ostia. Three chromosomes of the haploid set of eleven can be seen at the lower left. Portions of polar bodies I and II are visible outside the egg. × 970.

FIGURE 4. Two interphase nuclei in an egg from the coelom kept for 40 hours in Niu-Twitty solution. × 770.

TABLE II

Comparison of number of advances past Metaphase II in eggs with no jelly coats and eggs having jelly coats in ligation experiments

	Metaphase II	Later than Metaphase II	Total
With jelly	229	0	229
Without jelly	130	7	137
Totals	359	7	366

centrally located within the mass apparently never came in contact with the secretory epithelium and obtained no jelly. The eggs from region C in which advances were observed were of this type.

The frequency of advance past metaphase II in jelly-covered and non-jelly-covered eggs is given in Table II. Eggs in stages earlier than metaphase II and eggs which were probably not actually delayed by a ligature (*e.g.,* coelomic eggs taken from an animal whose oviducts were ligated toward the middle or posterior part) are not included. Statistical analysis of the distribution gives $P = < .01$.

In vitro experiments: Information as to the stages found in the 254 eggs of the four types kept *in vitro* is given in Table III. No advances past metaphase II occurred in jelly-covered eggs, and only one doubtful advance occurred in the eggs from which the jelly had been removed. Advances were found in 22% of the eggs never exposed to jelly. Comparison of the frequency of advance in eggs exposed to jelly (including those from which the jelly was removed) with those never exposed to it gives $P = < .001$.

TABLE III

Stages in eggs maintained in vitro (24 hours except in cases noted)

Type of egg	First meiotic division	Metaphase II	Anaphase II or Telophase II	Meiosis complete, condensed chromosomes or none found	Pronucleus or pronuclei	Post-meiotic "mitosis"	Total
Coelomic	0	73 (three for 12 hrs. only)	6 (one for 12 hrs. only)	1	2 (one for 12 hrs., one for 40 hrs.)	11	93
Ostial	0	8	2	1	0	0	11
Oviducal, with jelly	0	103 (four for 12 hrs., eleven for 48 hrs.)	0	0	0	0	103
Oviducal, jelly removed	0	46 (one for 12 hrs., eight for 48 hrs.)	1?	0	0	0	47
Totals		230	9	2	2	11	254

The solution in which the eggs were kept seemed to have no effect on the frequency of advance nor on the "normality" of the divisions, although eggs kept in Ringer's solution could usually be distinguished microscopically from those kept in Holtfreter's or Niu-Twitty solution by their "more dense" appearance. Also, the amount of fluid surrounding the egg seemed to be of little, if any, importance.

In eleven of the twenty coelomic eggs in which advance occurred, post-meiotic mitotic activity was occurring. Such "mitoses" often occurred well beneath the egg surface, as in a cleavage division, in contrast to meiotic divisions, which usually occur at the surface. In several instances it appeared that a haploid set of monads was being randomly distributed to the poles, since, even at anaphase, the total number of chromosomes on the spindle was only the haploid number. There were no asters nor was there any evidence of attempted cytoplasmic division. Such "mitosis" probably accounts for the "pronuclei" which were seen in one egg (Fig. 4).

Discussion

There are two general alternatives which may be suggested as possible explanations of the source of meiotic arrest in the amphibian egg. One is that the source is within the egg itself, the other is that the block is somehow imposed upon the egg through some aspect of the environment. The former seems to be the explanation in eggs of *Habrobracon,* where von Borstel (1957) has found evidence that blockage is under genetic control. A third possibility is that a combination of internal and external factors is involved. Not nearly enough is known of either the physiology of the amphibian oocyte or of its environment to allow a ready choice between the alternatives.

Probably the explanation most attractive at present is that proposing a blocking mechanism intrinsic to the egg. The results of the present study should be examined from this point of view. If this is done, certainly one feature of the data seems to agree strongly with the hypothesis of an intrinsic blocking mechanism, and that is the fact that the great majority of eggs which had progressed as far as the second division had not proceeded beyond metaphase of that division. This was true even of non-jelly-covered eggs, the only type in which the block was evaded. The implication is that the process normally stops at this stage, and that, rather infrequently, it is capable of proceeding to completion. If nothing else, it appears that there is a lengthy pause at metaphase. However, in many cases there was little indication as to how long the divisions had been in progress, and undoubtedly some of the eggs had only recently reached metaphase II, and could not have been expected to have advanced beyond that stage. If intrinsic blockage is assumed, however, then what mechanism could account for the evasion of the block in the eggs of this study? It is possible that degenerative changes within the eggs somehow triggered the completion of the second division, but if this is the case, the jelly deposited by the oviducts must protect against such changes or the effects of such changes, since no jelly-covered egg showed advance. Also, eggs which are laid, but not fertilized, will degenerate in their jelly coats without advancing past the normal stage of blockage.

Under the assumption of intrinsic blockage, perhaps the most acceptable hypothesis for explaining the present results is that some aspect of the environment, either

the natural coelomic or ostial environment or the artificial ones, was effective in stimulating completion of the division, perhaps through stimulation of the division apparatus directly or through a general stimulation of some aspect of the metabolism of the egg. Again, however, the jelly coats seem to protect, this time against a possible external stimulatory effect, since jelly-covered eggs which escaped from the oviducts back into the coelom, or which were kept *in vitro* for as long as 48 hours, were never more advanced than metaphase II.

The present results should also be viewed in the light of the possibility that normal meiotic blockage is a function of some factor of the environment of the egg, and not of the egg itself. Under this hypothesis, meiosis might be considered to be a process which, once having been initiated, would, in the absence of outside interference, continue to completion. If an outside inhibitor is considered, the oviducal secretions, specifically the jelly layers deposited about the oocyte, seem to be especially suspect as a source of inhibition. Previous workers (Bataillon and Tchou-Su, 1930; Dalcq *et al.*, 1936) have considered the oocytes of anuran amphibians to be "intoxicated" with carbon dioxide, presumably related to the secretions of the oviducts. Such an "intoxication" might itself be the cause of meiotic blockage, which would perhaps be thought of as an indirect effect of the oviducal jelly.

There is also the possibility of a more direct effect of the oviducal secretions. The first division is usually completed and the second division spindle is usually formed in the anterior part of the oviduct (Humphries, 1956). The division spindle is located with one pole at the egg surface, where it might be unusually sensitive to an outside effect. These facts make the idea of a possible effect of oviducal secretion worth consideration, especially in the light of the results of the present study. The lack of advance in jelly-covered eggs is itself important in this consideration, but the fact that only one doubtful case of escape from blockage occurred in the eggs from which the jelly was removed before their stay *in vitro* suggests that mere exposure to the jelly, not necessarily its presence, may somehow be effective in preventing advance. Recent studies on the histochemistry of the oviducts of urodeles (Kambara, 1956a, 1956b, 1957a, 1957b; Humphries and Hughes, 1959) have shown the presence of acid mucopolysaccharide in the anterior regions of the oviducts, while Minganti (1955) has reported a heparin-like anticoagulant activity of amphibian egg jelly. Heilbrunn and his co-workers, as well as others, have repeatedly shown that compounds of this type are capable of antimitotic activity and that they are of widespread occurrence (see especially Heilbrunn, 1956, and Heilbrunn *et al.*, 1957). Others, working with eggs of the sea urchin, have apparently implicated mucopolysaccharides in general inhibitory activity prior to fertilization (Runnström and Immers, 1956). Certainly the present findings require a serious consideration of the possibility of a direct or indirect effect of the oviducal secretions.

The exploration of the problem of meiotic blockage thus far does not allow a choice between the major alternative explanations. It is apparent that the jelly layers are of significance with regard to blockage under the conditions of these experiments, and they may be involved, as well, in blockage as it occurs under natural conditions. However, the jelly may be important only as an insulator from the environment. Were the jelly the source of the meiotic block, one would expect

all eggs unexposed to jelly to complete meiosis if given ample time and suitable environment. If we assume that these conditions were met by the *in vitro* experiments reported here, in which most such eggs showed arrest, then it is not possible to maintain that the jelly is the sole factor in meiotic arrest, although it may play an important ancillary role, such as the maintenance of an already established block.

Summary

1. The oviducts of the newt, *Triturus viridescens,* were ligated at several levels to retain oocytes from 48 to 72 hours in the coelom or in certain regions of the oviducts. Eggs were collected and studied to determine the stage of meiosis, with the finding that advances past metaphase II, the normal stage of arrest, occurred at a low frequency, but were confined to eggs never exposed to the jelly secreted by the oviducts.

2. Twenty-two per cent of the eggs removed from the coelom or ostium of the oviduct and kept *in vitro* advanced past metaphase II, but eggs with jelly coverings did not advance. One doubtful advance was observed in an egg from which the jelly had been removed prior to its stay *in vitro*. Some "advanced" eggs exhibited post-meiotic "mitotic" activity.

3. The general problem of meiotic blockage in the egg is discussed. The jelly secreted by the oviducts is of significance with regard to meiotic arrest under the conditions of the experiments reported, and may play some role in normal arrest. The evidence does not, however, allow a decision as to the source of meiotic inhibition.

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